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Loss of the catalytic subunit of the DNA-dependent protein kinase in DNA double-strand-break-repair mutant mammalian cells

(Ku protein/*scid/scid* mouse/DNA repair)

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ABSTRACT **The DNA-dependent protein kinase (DNA-PK) consists of three polypeptide components: Ku-70, Ku-80, and an ≈350-kDa catalytic subunit (p350). The gene encoding the Ku-80 subunit is identical to the x-ray-sensitive group 5 complementing gene XRCC5. Expression of the Ku-80 cDNA rescues both DNA double-strand break (DSB) repair and V(D)J recombination in group 5 mutant cells. The involvement of Ku-80 in these processes suggests that the underlying defect in these mutant cells may be disruption of the DNA-PK holoenzyme. In this report we show that the p350 kinase subunit is deleted in cells derived from the severe combined immunodeficiency mouse and in the Chinese hamster ovary cell line V-3, both of which are defective in DSB repair and V(D)J recombination. A centromeric fragment of human chromosome 8 that complements the *scid* defect also restores p350 protein expression and rescues *in vitro* DNA-PK activity. These data suggest the *scid* gene may encode the p350 protein or regulate its expression and are consistent with a model whereby DNA-PK is a critical component of the DSB-repair pathway.**

The molecular mechanisms by which mammalian cells detect and repair DNA double-strand breaks (DSBs) have recently begun to be elucidated through the study of mutant cell lines that are deficient in DSB repair. The molecular defect affecting DSB repair in cells from the x-ray-sensitive complementation group 5 can be rescued by a human gene, designated XRCC5, that maps to the q35 region of chromosome 2 (1, 2). In addition to the DSB-repair defect, group 5 cells are deficient in V(D)J recombination. The DSB repair and V(D)J recombination defect of group 5 cells can be rescued (3, 4) by expression of the gene encoding the 86-kDa subunit of the human autoantigen protein Ku (5).

These results suggest that the Ku-80 protein is an essential component of both DSB repair and V(D)J recombination pathways. Clues to how the Ku-80 protein may participate in these processes may be revealed through examination of the *in vitro* biochemical properties of the Ku protein. The 86-kDa subunit of the protein (Ku-80) associates with a second polypeptide, Ku-70 (6), to form a heterodimeric complex that binds with high affinity to the ends of double-stranded DNA (3, 7–11) and to single- to double-strand DNA transitions (12). In addition, in the presence of DNA the Ku-70/80 complex can bind to and stimulate the activity of an ≈350-kDa protein kinase catalytic subunit (p350) (13–16). The trimeric complex consisting of Ku-70, -80, and p350 constitutes the DNA-dependent protein kinase (DNA-PK) (17, 18).

Loss of Ku-80 function in the group 5 cells disrupts the DNA-binding activity of the Ku complex (19–21). In the absence of the Ku complex, formation of the DNA-PK holoenzyme is inhibited (S.R.P., F. Chen, and D.J.C., unpublished work). If the primary function of the Ku complex is to serve as an activator of the p350 kinase subunit, then the loss of DNA-PK activity may cause the underlying DSB repair and V(D)J recombination defects of the Ku-80 mutant cells. If this is true, then disruption of the gene encoding the p350 kinase would result in a phenotype similar to the Ku-80 mutants and may be responsible for repair defects observed in cells from other DSB-repair complementation groups. On the basis of this rationale, we investigated the status of the p350 protein in other cells deficient in DSB repair and V(D)J recombination that fall outside the XRCC5 complementation group. We find that the p350 subunit of DNA-PK is absent in cells derived from the severe combined immunodeficiency (SCID) mouse (22) and in the Chinese hamster ovary (CHO) cell line V-3 (23), both of which share defects in DSB repair (23–25) and V(D)J recombination (26, 27). Complementation of the SCID cell phenotype by a DNA fragment encompassing the centromeric region of human chromosome 8 restores p350 protein expression and DNA-PK activity. These results suggest that the *scid* gene encodes the p350 protein and that DNA-PK may be an essential component of DSB repair and V(D)J recombination processes.

MATERIALS AND METHODS

Cell Culture. The human diploid fibroblast cell line HSF55 and the mouse and CHO cells were cultured in 162-cm^2 dishes under subconfluent conditions using α-minimum essential medium/10% heat-inactivated calf serum/penicillin/streptomycin in a humidified incubator at 37°C with a 5% CO_2/95% air atmosphere. The cell line SC(8)-10 was cultured under these same conditions in the presence of G418 at 400 μg/ml to maintain the human chromosomal fragment.

Cell Extract Preparation. Cells were harvested by scraping into an ice-cold phosphate-buffered saline (PBS) solution, pelleted by centrifugation at 600 × *g* for 2 min, and washed two times with 15 ml of ice-cold PBS. Hypotonic cell extracts were prepared by swelling the washed cells in four packed cell volumes of hypotonic lysis buffer (10 mM Tris·HCl, pH 7.9/10 mM KCl/1.5 mM $MgCl_2$) containing 1 mM dithiothreitol, phenylmethylsulfonyl fluoride at 20 μg/ml, aprotinin at 1 μg/ml, leupeptin at 1 μg/ml, pepstatin A at 1 μg/ml, soybean trypsin inhibitor at 10 μg/ml, and 0.1 mM sodium orthovanadate for 10 min at 0°C. The swelled cells were then lysed by

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Abbreviations: DSB, double-strand break; DNA-PK, DNA-dependent protein kinase; SCID, severe combined immunodeficiency; CTD, carboxyl-terminal domain.

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Dounce homogenization, and the cell suspension was centrifuged at 3000 × *g* to pellet the nuclei. The resultant supernatant represents the hypotonic lysate. A high salt cell extract was prepared by resuspending the pelleted nuclei in four packed cell volumes of lysis buffer [50 mM Tris·HCl, pH 7.9/420 mM KCl/12.5 mM $MgCl_2$/1 mM EDTA/20% (vol/vol) glycerol/10% (wt/vol) sucrose] containing 1 mM dithiothreitol, phenylmethylsulfonyl fluoride at 20 μg/ml, aprotinin at 1 μg/ml, leupeptin at 1 μg/ml, pepstatin A at 1 μg/ml, soybean trypsin inhibitor at 10 μg/ml, and 0.1 mM sodium orthovanadate for 30 min at 4°C with stirring. Both the nuclear and hypotonic extracts were dialyzed against TM buffer (50 mM Tris·HCl, pH 7.9/12.5 mM $MgCl_2$/1 mM EDTA/20% glycerol) containing 100 mM KCl, phenylmethylsulfonyl fluoride at 20 μg/ml, aprotinin at 1 μg/ml, leupeptin at 1 μg/ml, pepstatin A at 1 μg/ml, soybean trypsin inhibitor at 10 μg/ml, 0.1 mM sodium orthovanadate, and 1 mM dithiothreitol for 16 hr at 4°C and then clarified by centrifugation at 16,000 × *g*. Protein concentration of the clarified extracts was determined by Bradford analysis, and the solutions were adjusted to 4 mg/ml. The p350 protein was found primarily in the hypotonic fraction of the rodent cells, and the Ku-70 protein was equally distributed in the hypotonic and high salt fractions. Whole-cell extracts were prepared by combining equal amounts of protein from the hypotonic and high salt fractions.

Antibodies and Immunoblotting. Cell lysates were boiled in SDS/PAGE sample loading buffer and resolved by SDS/PAGE. For p350 analysis a 6.5% gel was used, and for Ku-70 analysis an 8% gel was used. Proteins were transferred to nitrocellulose using a semidry apparatus, blocked for 30 min with a solution of 5% Carnation nonfat milk in 1× TTBS (10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.1% Tween 20) and incubated with primary antibody at room temperature for 1 hr. The blots were then washed three times with 250 ml of 1× TTBS and then incubated with a 1:5000 dilution of a horseradish peroxidase-conjugated secondary antibody (Amersham) in 1× TTBS for 2 hr at room temperature. The antibody wash step was then repeated, and immunoreactive proteins were visualized by chemiluminescent detection using epichemiluminescence reagents (Amersham). The anti-p350 monoclonal antibodies 18-2 and 42-26 and the anti-Ku-70 antibody NH310 were obtained from mouse ascites fluid and used for immunoblotting at a concentration of 1 μg/ml.

Indirect Immunofluorescence Microscopy. Cell monolayers cultured on chambered glass slides were rinsed once with PBS and then fixed to the slide with ice-cold acetone for 3 min on a bed of ice. Fixed cells were blocked with a 5% solution of bovine serum albumin in 1× TTBS for 30 min at room temperature. After a brief PBS rinse the slides were incubated with a solution (10 μg/ml) of the anti-p350 monoclonal antibody 18-2 in 1× TTBS/5% bovine serum albumin for 60 min at room temperature. After three washes with 1× TTBS the slides were incubated with solution (1 μg/ml) of a goat anti-mouse-fluorescein conjugated antibody (Oncogene Science) in 1× TTBS/5% bovine serum albumin for 60 min at 37°C in the dark. After the secondary antibody incubation the slides were washed three times with 1× TTBS, once with PBS, allowed to air-dry, and then mounted using Vectashield (Vector Laboratories) mounting medium containing propidium iodide at 17 μg/ml. Photographic images were obtained by using a Neofluar 40× lens mounted on an Axioplan microscope (Zeiss) and Kodak Ektar 1000 color print film (Eastman Kodak).

DNA-PK Assays. DNA-PK activity was measured by incubating 4 μg of whole-cell extract with 90 ng of a GAL4–carboxyl-terminal domain (CTD) fusion protein substrate (28) in reactions containing no DNA or 1 ng of a DNA fragment containing five GAL4 binding sites, as described (29). Identical reactions were also performed in the absence of the GAL4–CTD fusion protein. Kinase reactions were initiated by the addition of 12.5 μM [γ-^{32}P]ATP (8 Ci/mmol; 1 Ci = 37 GBq) and incubated at 30°C for 30 min. Kinase reactions were terminated by boiling in SDS/PAGE sample buffer, and the products were analyzed as described in the figure legends.

RESULTS

To assess whether alteration of p350 protein expression may be associated with defects in DSB repair and V(D)J recombination, we compared p350 protein levels in murine SCID cells and CHO V-3 cells with wild-type mouse and CHO cells. Immunoblot analysis was done by using two monoclonal antibodies directed against the human p350 protein (17) with a cell extract prepared from human diploid fibroblasts (HSF55) as a control (30) (Fig. 1*A*, lane a). We also detected p350 protein in extracts prepared from the wild-type mouse cell line M5S (31) but not in extracts prepared from the *scid* mouse cell line SCVA2 (32) or in primary lung fibroblasts derived from the SCID mouse (Fig. 1*A*, lanes b, c, and e). However, expression of the p350 protein was detected in extracts prepared from cell line SC(8)-10 (Fig. 1*A*, lane d), a radiation-resistant subclone of SCVA2 that contains a centromeric portion of human chromosome 8 (32). The p350 protein was also detected in extracts prepared from the wild-type CHO cell line AA8 (23) but was absent in extracts prepared from the CHO V-3 cell line (Fig. 1*A*, lanes f and g). Fusion of the V-3 cell with HSF55 rescued the radiosensitive phenotype (data not shown) and restored expression of the p350 protein (Fig. 1*A*, lane h). As a control, we measured the expression of the Ku-70 protein in each of these extracts. Immunoblot analysis using the Ku-70

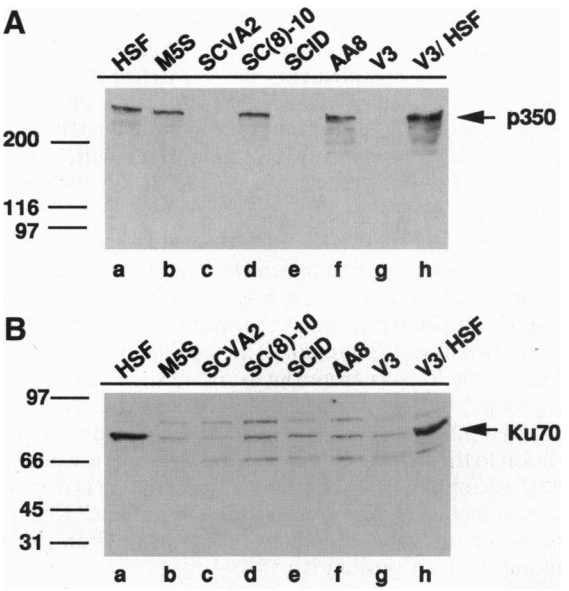

FIG. 1. Immunoblot analysis of p350 and Ku-70. Hypotonic cell extracts of the human diploid fibroblast HSF55 (lane a), wild-type mouse M5S (lane b), SCID mutant mouse SCVA2 (lane c), SCID mouse–human chromosome 8 hybrid SC(8)-10 (lane d), primary *scid* fibroblast (lane e), wild-type CHO AA8 (lane f), CHO mutant V-3 (lane g), and a V-3–HSF55 hybrid (lane h) were resolved by SDS/PAGE and transferred to nitrocellulose as described. (*A*) The p350 protein was detected by immunoblotting immobilized proteins with an equal mixture of the anti-p350 monoclonal antibodies 18-2 and 42-26 as described. The total amount of protein analyzed for the HSF55, SC(8)-10, and V-3/HSF extracts was 20 μg. For the other cell extracts 80 μg was used. Arrow indicates the position of the p350 protein. The position of prestained molecular mass markers is indicated in kDa. (*B*) Ku-70 protein was detected by immunoblotting with the anti-Ku-70 monoclonal antibody NH310 as described. Twenty micrograms of material was analyzed from the HSF55 and V-3/HSF extracts. For the other cell extracts 80 μg of material was used. Arrow indicates the position of Ku-70. The position of prestained protein molecular mass markers is indicated in kDa.

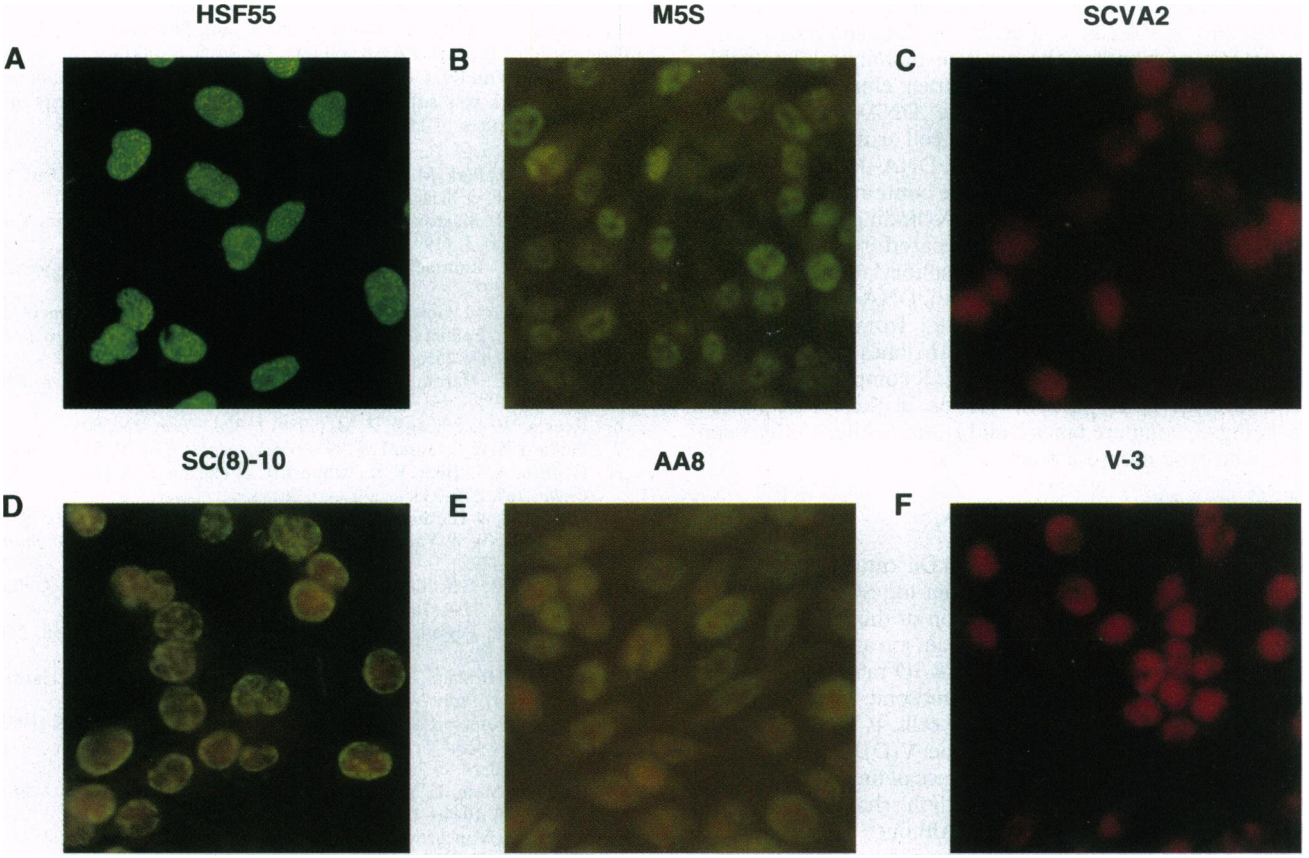

FIG. 2. Indirect immunofluorescent visualization of p350. Cell monolayers were stained using the anti-p350 antibody 18-2 (17) and visualized as described. The fluorescein isothiocyanate secondary antibody stains the p350 protein green. Cell nuclei are stained red by propidium iodide. (*A*) Human diploid fibroblast HSF55. (*B*) Wild-type mouse cell M5S. (*C*) SCID mutant mouse cell SCVA2. (*D*) SCID mouse–human chromosome 8 hybrid cell SC(8)-10. (*E*) Wild-type CHO cell AA8. (*F*) CHO mutant cell V-3. Similar results were obtained under identical conditions using the anti-p350 antibody 42-26 (17) (data not shown).

monoclonal antibody NH310 (33) showed that Ku-70 protein levels in each of the rodent cell extracts were similar (Fig. 1*B*, lanes b–g). Expression of the Ku-70 protein in the human and the V-3–human hybrid cells was greater than that found for the rodent cells (Fig. 1*B*, lanes a and h). These data agree with previous reports that showed Ku-like DNA-binding activity was not affected by the *scid* mutation (19) or by the mutation affecting the CHO V-3 cells (19, 20).

The expression and subcellular localization of the p350 protein in these cells were examined by indirect immunofluorescence. The p350 protein was visualized against a background of propidium iodide-stained cell nuclei using the anti-p350 monoclonal antibody 18-2 (17) and a fluorescein isothiocyanate-conjugated secondary antibody. The p350 protein was primarily found to be localized to the cell nucleus in the human fibroblasts (Fig. 2*A*), as was shown previously for HeLa cells (17). In the SC(8)-10 cells the p350 protein was also localized to the cell nucleus (Fig. 2*D*). Although the fluorescent signal was weaker, we found that p350 was also found primarily in the nuclei of the wild-type mouse and CHO cells

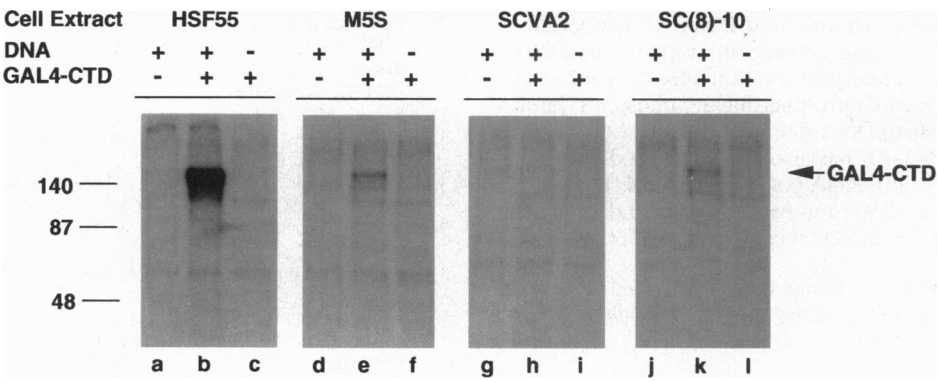

FIG. 3. Determination of DNA-PK activity in wild-type and SCID mutant cell extracts. Phosphorylation of a GAL4–CTD fusion protein substrate was determined for cell extracts prepared from human diploid fibroblast HSF55 cells, wild-type mouse M5S cells, SCID mutant mouse cells SCVA2, and the SCID mouse–human chromosome 8 hybrid cells SC(8)-10 as described. Reactions were done with or without DNA and GAL4–CTD substrate as indicated, and products of the reactions were analyzed by SDS/7.5% PAGE and visualized by autoradiography. Arrow indicates the position of the hyperphosphorylated form of the CTD–fusion substrate. The position of protein molecular mass markers is indicated in kDa.

(Fig. 2 *B* and *E*). No nuclear fluorescent signal was obtained for either the SCVA2 or V-3 cells (Fig. 2 *C* and *F*).

To determine whether the absence of immunodetectable p350 protein in the SCVA2 cell extract eliminates cellular DNA-PK activity, we compared the DNA-PK activity in wild-type and SCID mutant mouse cell extracts. DNA-PK activity was assayed by measuring the DNA-dependent phosphorylation of a recombinant substrate containing the CTD of RNA polymerase II fused to the DNA-binding domain of the yeast GAL4 protein (28). Extracts prepared from HSF55 cells showed a strong DNA-stimulated phosphorylation of the CTD substrate (Fig. 3, compare lanes b and c). DNA-PK activity was also observed when extracts prepared from the wild-type mouse extract were used (Fig. 3, compare lanes e and f) but was absent in the SCVA2 cell extracts (Fig. 3, compare lanes h and i). The level of CTD phosphorylation in the SC(8)-10 cell extract (Fig. 3, compare lanes k and l) was similar to that seen for the wild-type mouse extract.

DISCUSSION

In this report we show that the 350-kDa catalytic subunit of DNA-PK is absent in cells derived from the SCID mouse and from the CHO cell line V-3. Expression of the p350 protein is restored in SCID mouse cells that contain a fragment of human chromosome 8 that complements the SCID radiosensitive phenotype (32). The presence of the chromosome 8 fragment also rescued DNA-PK activity in the SCID cells. It has been shown previously that V-3 cells have the same V(D)J recombination phenotype as SCID cells (26). The absence of the p350 protein in both the SCID and V-3 is consistent with the shared defect being caused by changes to the p350 gene. Although these data lend evidence that the *scid* gene encodes the p350 protein, it is also possible that the *scid* gene defect affects the expression of p350 protein through a trans-acting mechanism.

That the absence of p350 also affects both DSB repair and V(D)J recombination makes sense when considered in the context of DNA-PK function. We propose that in both the Ku-80 and the p350 mutant cells the root cause for the mutant phenotypes is the loss of DNA-PK complex binding at sites of double-strand DNA breaks. The Ku protein likely functions to recruit p350 to these sites and potentiate p350 kinase activity. This action may serve to colocalize DNA-PK along with other proteins involved in DNA-repair activities, some of which may be activated via DNA-PK phosphorylation. Another possibility is that the p350 kinase modifies the activity of the Ku complex and that this modification is necessary for DNA-repair activities. Indeed, both subunits of the Ku protein are phosphorylated by DNA-PK *in vitro* (34, 35). Furthermore, the Ku complex has an intrinsic ATPase activity that may be regulated by DNA-PK phosphorylation (35).

It has been proposed previously that DNA-PK may participate in initiating a signaling cascade in response to DNA damage (36). The Ku-80 and p350 mutant cells now offer an exceptional means to identify proteins that are phosphorylated by DNA-PK in response to DNA-damaging agents. In addition to these processes, DNA-PK has also been proposed to play a role in transcription by both RNA polymerase I and II (29, 37). Use of the Ku-80 and p350 mutant cells should also aid in delineating how DNA-PK affects these biochemical pathways.

Note Added in Proof. After this manuscript was accepted for publication, we learned that the following paper reporting similar findings was accepted for publication (38).

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